REMARKS

Claim Status

Claims 1-40 are pending in this application. Claims 6, 10-13, 21, 25-28, 33, and 37-40 have been withdrawn. Upon entry of this Amendment and Response, claims 1-5, 7-9, 14-20, 22-24, 29-32, and 34-36 will be under examination.

Subject Matter Claimed

The claims of this application are directed to *a method for improving gene therapy* by increasing the level of expression of a recombinant protein *in vivo*, the protein having been expressed from an expression vector which has been introduced into the cells, by administering an active site-specific chaperone for the protein. Dependent claims recite that the protein is a lysosomal enzyme, particularly a lysosomal enzyme associated with a lysosomal storage disease, and more particularly that the enzyme is α-galactosidase or glucocerebrosidase. Other dependent claims recite that the vector is a viral vector, and that the chaperone is a reversible competitive inhibitor of the enzyme.

Rejections Under 35 U.S.C. § 103-Obviousness

Claims 1-5, 7-9, 14-17, 19, 20, 22-24, 29-32, and 34-37 remain rejected over U.S. patent 6,066,598 to Yew et al. ("Yew") taken together with commonly-owned U.S. Patent 6,274,597, to Fan et al. ("the '597 patent"). The '597 patent discloses a method for treating Fabry disease by administering a competitive inhibitor as a chaperone for α-galactosidase A (α-Gal A). According to the specification, the chaperone enhances (*i.e.*, increases) the activity of endogenous mutant α-Gal A in cells from Fabry disease patients, and enhances (*i.e.*, increases) activity of a wild-type α-Gal A overexpressed in a transgenic mouse. Gene therapy is neither disclosed nor suggested. Yew discloses a method of providing active α-Gal A to cells of an individual having an α-Gal A deficiency, such as a Fabry disease patient, by administering a vector containing a functional α-Gal

A gene to (*i.e.*, gene therapy). Chaperone therapy is neither disclosed nor suggested, nor does Yew suggest any method which pertains to correcting the underlying genetic defect.

Claims 17 and 18 stand rejected as obvious as allegedly being unpatentable over Yew in view of the '597 patent, further in view of Hendricks (*Blood.* 2000; 96:845a; "Hendricks"). Hendricks discloses a method for indirect gene therapy by administering human mesenchymal stem cells which have been transduced with a vector expressing α-Gal A. Hendricks does not teach or suggest combining gene therapy with the chemical chaperone therapy of the '597 patent. Because the combination of Yew with the '597 patent fails to suggest the claimed invention, much less provide a reasonable expectation of success at achieving the claimed invention, the further addition of Hendricks would not affect patentability of claims 17 and 18.

According to the Examiner, it would have been obvious to co-administer a vector containing a replacement gene and a chaperone for the protein expressed from the replacement gene from the combined teachings of the '597 patent and Yew, since one of ordinary skill in the art would have expected that a combination of increasing a patient's endogenous mutant α-Gal A using a chaperone ('597), and replacing the deficient gene with a gene encoding a functional α-Gal A (Yew and Hendricks), would provide even more α-Gal A activity than either method alone (Final Office Action, p. 5). In the Examiner's subsequent Advisory Action, the Examiner elaborates by stating "...it would have been obvious to treat Fabry's disease with both of the patented methods simultaneously, in order to obtain the benefits of each method."

This rejection is respectfully traversed for the following reasons.

In order to make a *prima facie* case for obviousness, there must be some motivation to combine the references that create the case of obviousness. *In re Rouffet*, 149 F.3d 1350, 47 USPQ2d 1453 (Fed. Cir. 1998). The motivation often comes from the teachings of the pertinent references (*Akamai Technologies, Inc. v. Cable & Wireless Internet Services, Inc.*, 344 F.3d 1186, 68 USPQ2d 1186 (Fed. Cir. 2003). However, the motivation can also be found in the knowledge of persons of ordinary skill in the art, or in the nature of the problem to be solved. *Id.* Despite this, the

range of sources available does not diminish the requirement for actual evidence. *In re Dembiczak*, 175 F.3d 994, 50 USPQ2d 1614 (Fed. Cir.1999). In addition, obviousness requires a determination of whether there was something in the prior art *as a whole* to suggest the ability of making the combination. *Continental Can Company USA, Inc. v. Monsanto Co.*, 948 F.2d 1264, 20 USPQ2d 1946 (Fed. Cir. 1991) (emphasis added).

In the present case, the Examiner has asserted that the motivation to combine Yew and the '597 patent and Hendricks can be found in the knowledge of those of ordinary skill in the art in the field of treating Fabry disease (Final Office Action, p. 5). However, none of the Yew, the '597 patent, or Hendricks explicitly or implicitly suggest any type of combination therapy, so the motivation cannot be found in the references themselves. On the contrary, Yew, Hendricks, and the '597 patent references, taken *as a whole* for what they disclose, would not have motivated an ordinarily skilled person to combine chaperone therapy with gene therapy to treat a Fabry patient. This is because the '597 patent discloses a paradigm-shifting approach to the treatment of genetic diseases that provides an alternative to protein replacement or gene therapy. The concept of fixing the patient's *endogenous mutant* protein using an *inhibitor* for the protein, which is already deficient, is an alternative approach to replacing the mutant protein or gene by *exogenous* enzyme replacement therapy (ERT) or gene therapy. The '597 patent is almost exclusively focused on correcting the underlying genetic defect in Fabry disease patients and discloses a mechanism which, if successful, precludes the need for ERT or gene therapy in patients whom chaperone therapy can be applied.

As of the filing date, there was no approved therapy for Fabry disease. Nevertheless, the skilled artisan would have known that even *small* increases in residual α-Gal A activity (to 30% of normal) could prevent major clinical manifestations resulting from the defect (see '597 at col. 3, ll. 45-52). See also Desnick et al., *The Metabolic and Molecular Bases of Inherited Disease*; Chapter 150, pp. 3733-3774, McGraw Hill Companies., 2001, page 3749 (10-40% of normal; relevant page attached).

The '597 patent discloses that administration of a chaperone produced very *large* increases in activity *in vivo* in tissues of transgenic mice expressing mutant α-Gal A (at least about 2-fold; see Example 10, beginning at the bottom of col. 8), the artisan would have had no reason to consider a combination with gene therapy to achieve a synergistic increase in the patient's endogenous α-Gal A and the gene therapy α-Gal A, since the patients could be efficaciously treated using a chaperone alone.[1] One of ordinary skill in the art would have recognized from the '597 patent *as a whole* that treating a disease associated with a mutation resulting in protein instability using a small molecule sugar analogue would **i)** be easily administrable (oral), **ii)** be inexpensive, **iii)** have high bioavailability (e.g., when the molecule is a soluble sugar such as deoxyglactonojirimycin), and **iv)** have the ability to penetrate difficult biological barriers and enter, e.g., renal podocytes and cardiomyocytes, which are cell types that a skilled artisan would have known have significant substrate accumulation in Fabry disease. Thus, knowing of the '597 chaperone method, one of ordinary skill in the art would have recognized that chaperone therapy could treat a significant population of Fabry patients who express a conformationally defective, but catalytically active, α-Gal A. It follows that such a skilled artisan also would have recognized that these characteristics would provide significant advantages over the other potential therapies known in the art, such as gene therapy or ERT, which are more invasive, less efficacious due to systemic delivery problems, and much more expensive to produce and administer than an oral tablet, particularly since the gene therapy would "guild the lilly," i.e., have no further patient benefit since chaperone therapy would achieve sufficient levels of defective protein activity.

On the other hand, patients who did not express a functional enzyme, or any enzyme, would be non-responsive to chaperone therapy. In these patients, enzyme replacement therapy or gene therapy are the only workable alternatives. Since chaperone therapy would have no effect on an inactive or absent endogenous gene, there would be no incentive to combine the '597 patent approach with gene therapy in these patients. Furthermore, Yew discloses that gene therapy in mice increased and sustained α-Gal A activity in all tissues evaluated to levels significantly *above* wild-

[1] Even without anticipating that the large 2 to 5-fold (i.e., 100-400%) increases observed in mice would present in humans, one would have recognized that even a smaller increase in humans (of e.g., 10-30%) would be sufficient to treat Fabry disease.

type (see Figure 10A). Thus, there is no benefit from adding chaperone therapy to the gene therapy of Yew, since according to Yew gene therapy provides adequate level of protein.

Thus, it is submitted that a skilled artisan knowledgeable in the field of treating Fabry disease, *i.e.*, knowledgeable of the clinical manifestations of the disease and having the best interests of the patient in mind, *and* having knowledge of the caveats of both gene therapy and ERT therapy, would not have any incentive to combine these approaches to treat Fabry, or any other disease. This is because such a knowledgeable artisan, realizing the differences between chaperone therapy and gene therapy, would have been motivated to choose chaperon therapy in patients having a functional mutant protein, e.g., a chaperonable α-Gal A, and gene therapy in patients with an inactive or absent protein, e.g., no chaperonable α-Gal A. A combination of the two would be needless, invasive, and cost prohibitive.

Simply because several alternative therapies are proposed for treating a disease does not mean that combinations of these therapies would be obvious to the skilled artisan when the facts argue otherwise. There must be some motivation to make the specific combination. In this case, there would have been no motivation to combine chaperone therapy and gene therapy because there would have been no need to achieve such a synergistic increase in α-Gal A activity, since according to both 'Yew and the '597 patent, levels of α-Gal A activity are sufficiently elevated with each respective treatment modality to make the other superfluous – in the subset of patients where the two can even be combined.

Indeed, the obviousness rejection seems to presume that that every patient who is treated using gene therapy because of a protein deficiency not only expresses some form of the protein that is deficient, but expresses a form that can be fixed by a chaperone (*i.e.*, is chaperonable). This is incorrect. Numerous protein deficiencies are caused by mutations, such as null mutations, that result in no mRNA and/or protein expression. The Examiner also presumes that every patient who *does* endogenously express some portion of a deficient protein would be able to benefit by chaperone therapy. This is also incorrect. Numerous protein deficiencies are caused by mutations which are nonsense mutations, deletion mutants, splicing mutants which severely truncate or distort

a protein. Other mutations which found at the protein's "active site" can inactivate the function of a protein even if the conformation could be stabilized. For example, in Fabry disease, although many of the hundreds of mutations identified are missense which affect the folding of α-Gal A, other mutations exist, including nonsense, catalytically inactivating missense, splicing, rearrangements, and deletions, some of which are not chaperonable by the '597 method (see Desnick, pp.3754-56; pages attached).

Thus, from the teachings of the '597 patent, a skilled artisan would not have expected that a chaperone would have any effect in a Fabry patient having a non-chaperonable mutant α-Gal A, since it either would not be able to interact with that enzyme (*e.g.*, a truncation), or it would not be able to restore protein activity (*e.g.*, a catalytic mutant).

Therefore, in the patients having non-chaperonable mutations, there can be no valid obviousness allegation according to the Examiner's reasoning, because one of ordinary skill in the art would have looked *only* to gene therapy or ERT to treat such a patient population, **and not** to chaperone therapy. It follows that, in seeking ways to *improve* gene therapy for treating the population of Fabry patients not expressing any α-Gal A (or expressing a form of α-Gal A which could not be rescued by a chaperone), the skilled artisan **would not** have looked for guidance to a reference which does not even disclose gene therapy, but, as a whole, discloses an alternative gene therapy, which alternative depends on the presence of an almost-full-length *and* catalytically active α-Gal A (the '597 patent). By contrast, one seeking to improve gene therapy in these patients would look only to references which, as a whole, are directed to improving gene therapy, such as Yew. (Although arguably even the Yew reference itself discourages looking any further than Yew itself to improve efficiency of gene therapy, because the vector disclosed in Yew "satisfies these needs"; see col. 3, ll. 6-38).

Thus, there would have been no motivation by one of skill in the art seeking to improve gene therapy in non-chaperoneable patients to combine the '597 patent with either Yew or Hendricks.

However, in each of the foregoing instances where the endogenous mutation resulted in a non-chaperonable protein, gene therapy would still be improved by the claimed method because the chaperone would stabilize, and thus, "increase the expression" of the functional replacement protein expressed from the administered vector. The present claims clearly encompass these embodiments as reflected in the instant specification as follows (using the published application as a reference; emphasis added):

Paragraph 0003: "Protein deficiency can be caused by a mutation in the coding gene which results in (i) **non-synthesis** of the protein; (ii) **synthesis of the protein which lacks biological activity**; or (iii) synthesis of a protein ...which cannot be appropriately processed to reach the native compartment of the protein.

Paragraph 0006: "Gene therapy involves replacing a defective or a **missing** gene...

Paragraph 0018: "However, effective in rescuing **conformationally defective** proteins, **[chaperones] cannot rescue proteins that are not ma[d]e**, e.g., as a result of a deletion mutation or nonsense mutation. Treatment of these conditions requires either replacing the protein (through protein replacement or gene therapy)."

Paragraph 0028: "...gene therapy also refers to the replacement of a defective gene encoding a defective protein, or **replacement of a missing gene**...

Paragraph 0031: "The term 'disorder characterized by a protein deficiency' refers to any disorder that presents with a pathology caused by **absent** or insufficient amounts of a protein. This term encompasses protein folding disorders, i.e., conformational disorders that result in a biologically inactive protein product."

Thus, while the method of the present claims encompasses both embodiments where the patient expresses a chaperonable protein and where the patient does not express the protein or does not express a chaperonable form of the protein, neither embodiment is obvious over the references cited as alleged by the Examiner. In the former instance, a skilled artisan would have been motivated to use chaperone therapy *in lieu* of gene therapy (or ERT) and in the latter, there would have be no motivation to combine the modalities because chaperone therapy would have provided for sufficient levels of protein rescue to make other treatment superfluous.

In view of the foregoing, it is submitted that the Examiner has not met the burden under §103 for *prima facie* obviousness. Specifically, the Examiner has failed to establish that there

would have been any motivation for a skilled artisan to combine the teachings of Yew with those of the '597 patent as of the February 2003 filing date. Instead, the Examiner relies on the disclosure in the present application to supply the missing suggestion to carry out the presently claimed method. Such hindsight reconstruction is impermissible.

Conclusion

Applicant respectfully requests entry of the foregoing Remarks in the file history of the application. Also in view of the foregoing Remarks, it is believed the pending application is in condition for allowance. Allowance of all of the claims is earnestly solicited.

If the Examiner believes that a telephone interview would facilitate resolution of any remaining issues, the Examiner is invited to contact the undersigned.

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Respectfully submitted,

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α-galactosidase A activity in cultured fibroblasts was partially purified and shown to have kinetic and thermostability properties similar to those of α-galactosidase A from normal fibroblasts.[114]

The 42-year-old Italian cardiac variant who presented with severe rheumatoid arthritis[111] had residual α-galactosidase A activity that was about 1 percent of normal in plasma and urine and was subsequently shown to have the common N215S α-galactosidase A mutation.[294] Immunoprecipitation with monospecific anti-α-galactosidase A antibodies demonstrated residual activity in granulocytes, lymphocytes, platelets, liver, and cultured fibroblasts ranging from 9 to 37 percent of the respective normal levels. The immunoprecipitated residual α-galactosidase A activity from fibroblasts had the same kinetic and physical properties as the immunoprecipitated enzyme from normal fibroblasts. Rocket immunoelectrophoresis studies demonstrated that the level of α-galactosidase A activity corresponded to the amount of enzyme protein. However, compared with the normal enzyme, the residual fibroblast α-galactosidase A was more thermolabile at pH 4.6 and 50°C, and significantly less stable at pH 7.4 and 37°C. This finding was consistent with the extremely low enzyme levels in plasma and urine. Interestingly, the levels of globotriaosylceramide in plasma and urinary sediment were both in the low heterozygote range. No lysosomal inclusions were observed in hepatocytes or Kupffer cells in percutaneously biopsied liver, although ultrastructural evidence of glycosphingolipid was observed in a kidney biopsy. These findings were consistent with the N215S mutation resulting in an unstable enzyme with normal kinetics. In this variant, it appeared that 10 to 40 percent of normal intracellular activity was sufficient to prevent the major clinical manifestations of the disease. In addition, the finding of only 1 percent of enzymatic activity in the plasma and low levels of plasma globotriaosylceramide suggested that circulating enzyme was not required to catabolize the plasma substrate.

Of the cardiac variants, the 54-year-old German cardiac variant described by von Scheidt et al.[120] has been extensively studied (see above, "Atypical Hemizygotes: The "Cardiac Variant"). This variant who had the M296V α-galactosidase A mutation had the typical lysosomal inclusions in myocytes from an endocardial biopsy, but no histologic or ultrastructural evidence of lysosomal substrate deposition in myocardial capillaries or in other biopsied tissues. The patient's plasma globotriaosylceramide concentration (4.2 nmol/ml) was only slightly higher than the levels in his normal brothers (3.6 and 4.0 nmol/ml), whereas his urinary sediment concentration was at the low end of the heterozygote range. His α-galactosidase A activities ranged from 2 to 22 percent of the respective normal mean values in granulocytes (lowest), urine, lymphocytes, cultured lymphoblasts and fibroblasts, and plasma (highest). The disease manifestations in this 54-year-old variant were limited to the myocardium; there was no histologic evidence of small-vessel involvement or clinically manifest renal disease. Apparently the mutant enzyme in this variant with the M296V mutation was sufficiently active in vivo to markedly limit substrate deposition. Similar biochemical findings have been observed in the other cardiac variants (see Table 150-2).

Of interest is the 26-year-old Japanese variant described by Kobayashi et al.[113] who had no detectable α-galactosidase A activity in cultured fibroblasts when assayed with synthetic substrates. However, loading studies conducted in cultured fibroblasts demonstrated hydrolysis of some of the exogenously supplied substrate, which suggested the presence of residual activity toward the natural substrate.

Finally, a 51-year-old asymptomatic Arab male described by Bach and colleagues[112] had 10 percent of normal α-galactosidase A activity in cultured skin fibroblasts and normal levels of globotriaosylceramide in his urinary sediment. Further enzymatic studies revealed that the residual enzymatic activity had a K_m value that was fourfold higher than normal and an increased stability.

Recently, we evaluated the α-galactosidase A CRIM status of six unrelated cardiac variants who had residual α-galactosidase activity and whose mutations were known (E59K, R112H, F113L, D244N, M296V, and R356W). Using an affinity-purified monospecific rabbit polyclonal antibody against purified recombinant human α-galactosidase A in an ELISA assay, all six variant males were CRIM-positive with CRIM levels of 1.1 to 7.8 percent of normal (Ashton-Prolla et al., personal communication).

THE MOLECULAR GENETICS OF α-GALACTOSIDASE A

Our understanding of human α-galactosidase A and Fabry disease has been advanced dramatically by the isolation of the full-length cDNA and entire genomic sequence encoding this lysosomal enzyme.[31–33] The full-length cDNA sequence provided the primary structure of the enzyme precursor, including the signal peptide. The subsequent isolation and sequencing of the entire chromosomal gene for α-galactosidase A allowed characterization of the structural organization and regulatory elements of this housekeeping gene.[32,33] *In situ* hybridization, restriction fragment length polymorphism (RFLP) studies, and the recent isolation and analyses of yeast artificial chromosomes (YACs) containing the α-galactosidase A gene and flanking markers[295–298] provided both genetic and physical mapping of the α-galactosidase A gene to the Xq22.1 region of the X chromosome. Initial studies of the mutations in unrelated Fabry families have identified a variety of lesions underlying the molecular genetic heterogeneity of this disease (see "Molecular Pathology of Fabry Disease" below). More accurate carrier diagnosis has become possible by identification of the specific lesions in families or by analysis of closely linked polymorphisms (see "Diagnosis" below). Eukaryotic expression of the full-length cDNA has resulted in the production of large amounts of active enzyme for characterization, crystallization, and trials of enzyme replacement therapy (see "Treatment" below). In addition, site-specific mutagenesis has been employed to provide information on the structure and function of the enzyme.

Gene Assignment

The locus for human α-galactosidase A was assigned to the X chromosome in 1970 when Kint convincingly demonstrated that the defective globotriaosylceramide hydrolysis in Fabry disease was due to the deficient activity of α-galactosidase A.[19] Gene-mapping studies of human-hamster somatic cell hybrids localized the α-galactosidase A gene to a region on the long arm of the X chromosome, Xq21 → q24.[299,300] Subsequently, the regional assignment was narrowed to Xq21 → q22 by using a series of somatic cell hybrids made from a human cell line with an X,2,15 translocation, 46X,t(X;2;15)(q22;p12;p12).[301] This localization was further refined to the region Xq22 by *in situ* hybridization using the radiolabeled cDNA as a probe.[34,302] In addition, the restriction fragments detected in genomic DNA by Southern hybridization, using the full-length cDNA as a probe, exactly matched those identified by restriction mapping of genomic clones for α-galactosidase A, which indicated the absence of closely related sequences or pseudogenes.[303] Thus, the *in situ* and Southern hybridization studies established the occurrence of a single X-chromosomal gene for human α-galactosidase A.

The gene assignment was localized to Xq22 by RFLP studies that indicated that the α-galactosidase A locus was closely linked to the anonymous X-chromosome probes DXS17 and DXS87, but not to DXS11 or DXYS1.[304–307] The Xq22 region spans approximately 10 to 12 megabases (Mb) of genomic DNA in the proximal long arm of the X chromosome. Detailed physical maps of this region have been constructed by pulsed-field gel electrophoresis and radiation hybrid mapping.[308] Recently, YACs containing the α-galactosidase A gene were isolated, and the order and distances of the flanking markers were defined.[295,296] These studies identified several CpG islands in the Xq22 region that predicted the presence of a high density of functional genes in this area.[295,297] More recently, an integrated STS/YAC physical, genetic, and transcript map of human X-chromosomal region

active sites.[364] On the basis of an analysis of the conserved aspartate residues in α-galactosidase A sequences from humans and other species, six highly conserved aspartate residues (D92, D93, D109, D165, D264, and D322) were identified. Expression constructs were synthesized in which each of the conserved aspartates was mutagenized to an asparagine. Each of these constructs transiently expressed normal or near-normal amounts of activity, and the expressed enzymes had the same K_m toward the artificial substrate, 4-methylumbelliferyl-α-*D*-galactopyranoside, with the notable exception of D93N, which exhibited no activity.[365] All of the transiently expressed mutant enzymes, including D93N, bound an immobilized substrate analogue and were competitively eluted with galactose. Further studies with the D93E construct, designed with the more conservative D-to-E substitution, resulted in an expressed enzyme with very low activity, indicating that residue D93 is a strong candidate for the nucleophile involved in the hydrolysis of α-galactosyl moieties by human α-galactosidase A.

Crystallography and X-Ray Diffraction. The stable expression of high levels of recombinant human α-galactosidase A (see above) permitted purification of large amounts of the enzyme for crystallization and preliminary diffraction analysis.[366] Diffractable crystals were grown by the "hanging drop" method of vapor diffusion. X-ray diffraction data collected from these crystals indicated that the crystals belong to the orthorhombic space group C2221 with cell dimensions of a = 93.8 Å, b = 141.1 Å, and c = 184.4 Å. The crystals diffracted to a resolution of 3 Å, and native data were collected to 3.5-Å resolution. Assuming a dimer per asymmetric unit of total molecular mass 110 kDa (with oligosaccharide structures), the Matthews coefficient was V_m = 2.77 Å/dalton, corresponding to a solvent content of 55 percent. The self-rotation function indicated the presence of a noncrystallographic twofold axis relating the dimeric subunits.

MOLECULAR PATHOLOGY OF FABRY DISEASE

The availability of the full-length cDNA[31,32] and genomic sequences[33] for human α-galactosidase A has permitted the investigation of the nature and frequency of the mutations causing Fabry disease. Techniques employed to analyze the molecular lesions include Southern and northern hybridization analyses, RNase A studies, PCR amplification of genomic DNA or reverse-transcribed mRNA for subsequent DNA sequencing by dideoxy nucleotide sequencing, cycle sequencing or automated sequencing, fluorescent chemical cleavage, fluorescence-assisted mismatch analysis, and single-strand conformational polymorphism analysis, among others. Such studies have demonstrated the variety of molecular lesions that cause Fabry disease, examples of which are discussed below. To date, over 150 mutations have been identified in the Human Gene Mutation Database (HGMD) (www.uwcm.ac.uk/uwcm/mg/hgmd0.html), in a gene which encodes a 421-residue subunit. Most of the mutations were "private," confined to individual pedigrees. Mutations that have been identified in more than one family mostly occurred at CpG dinucleotides. Alternatively, the families that share the same mutation are frequently found by haplotype analysis to be distant members of the same pedigree.[126,367] Characterization of these lesions has provided information on the nature and frequency of the mutations causing this disease as well as insights into the structure-function relationships of this lysosomal hydrolase. Moreover, the identification of the mutation in a given Fabry family permits the precise diagnosis of other family members by the use of mutation-specific detection methods, such as targeted DNA sequencing or allele-specific oligonucleotide hybridization. With the prospect of enzyme replacement therapy for Fabry disease, it is possible that knowledge of a patient's mutation may be important in devising genotype-specific treatment regimens. The major types of mutations causing Fabry disease are discussed below. Of the over 150 mutations recorded in the HGMD (Fig. 150-18), 71.6 percent were coding region missense or nonsense mutations, 6.5 percent were RNA processing defects, and 21.9 percent were large or small gene rearrangements. In addition, four complex mutations have been described.

The location of the reported mutations provides some insights into structure/function relationships of this enzyme. Mutations appear to occur randomly throughout the gene with some notable exceptions: there is only one reported mutation between the initiation codon and codon 31 (the region containing the signal peptide that controls targeting to the endoplasmic reticulum), and the 3′ end of exons 3 and 4 and the 5′ ends of exon 4 and 5 have no reported mutations. Interestingly, 23 percent of the nonsense mutations and 29 percent of the small insertions and deletions occurred in exon 7, thus indicating that the carboxy-terminal residues are critical for enzyme function, even though some of the nonsense mutations occurred at residues with a low degree of conservation, for example, Q386X and W399X. These observations may indicate that specific regions are either crucial to the function of this gene or that mutations in other regions may not generate a phenotype.

Coding Region Mutations. Approximately 70 percent of the mutations causing Fabry disease are missense or nonsense mutations. Of the coding region single-base alterations listed in the HGMD, 84 percent were missense mutations and 16 percent were nonsense mutations.[120,294,303,368–374] Point mutations have been described in all exons. However, exons 4 and 7 were relatively underrepresented with only 3 and 12 mutations respectively, while the remaining mutations were equally distributed among the other exons. Although most of the missense and nonsense mutations were detected in classically affected hemizygotes, several missense mutations (I91T,[372] R112H,[371] F113L,[372] P146S,[369] N215S,[294,368] M296V,[303] Q279E,[117,294] and R301Q[118,120]) were identified in asymptomatic or cardiac variants of Fabry disease.

RNA Processing Defects. Ten mutations altering the processing of the α-galactosidase A transcript have been described in classically affected hemizygotes.[294,372,374–380] Most occur at the 5′-splice donor sites, though four 3′-splice acceptor site alterations have been described. Many of these mutations have undergone further molecular characterization to demonstrate the RNA processing defect. For example, The IVS6^{+1} mutation was shown to result in exon skipping.[376] Northern hybridization analysis of affected males initially detected a shortened 1.25-kb α-galactosidase A mRNA, which was present at 50 to 60 percent of normal abundance. Heterozygotes had both the normal 1.45-kb and the shorter 1.25-kb transcript. RNase A protection analysis identified a deletion of about 200 bp that included all of exon 6. The exon 6 deletion resulted from a gt → tt substitution of the invariant 5′-donor splice consensus site of intron 6, causing abnormal splicing of the α-galactosidase A pre-mRNA. Similar studies have been carried out with the IVS3^{-1} mutation.[374]

Gene Rearrangements. The frequency of gene rearrangements in the α-galactosidase A gene causing Fabry disease was assessed in 165 unrelated patients by Southern hybridization analysis using the full-length α-galactosidase A cDNA as a probe[303] or by multiplex PCR amplification of the entire α-galactosidase A coding region.[381] The latter method determines the size of each of the seven exons in four PCR products that are simultaneously amplified and provides rapid screening for large (> 50 to 100 bp) insertions or deletions. By these two methods, one partial gene duplication and five partial gene deletions were identified,[303] a frequency of ~5 percent, which is similar to that reported for other X-linked diseases.[382] To date, no total gene deletions have been identified. Interestingly, a patient has been described with both Fabry disease and Duchenne muscular dystrophy, indicating that he had concurrent deletions in both Xp21 and Xq22.[383]

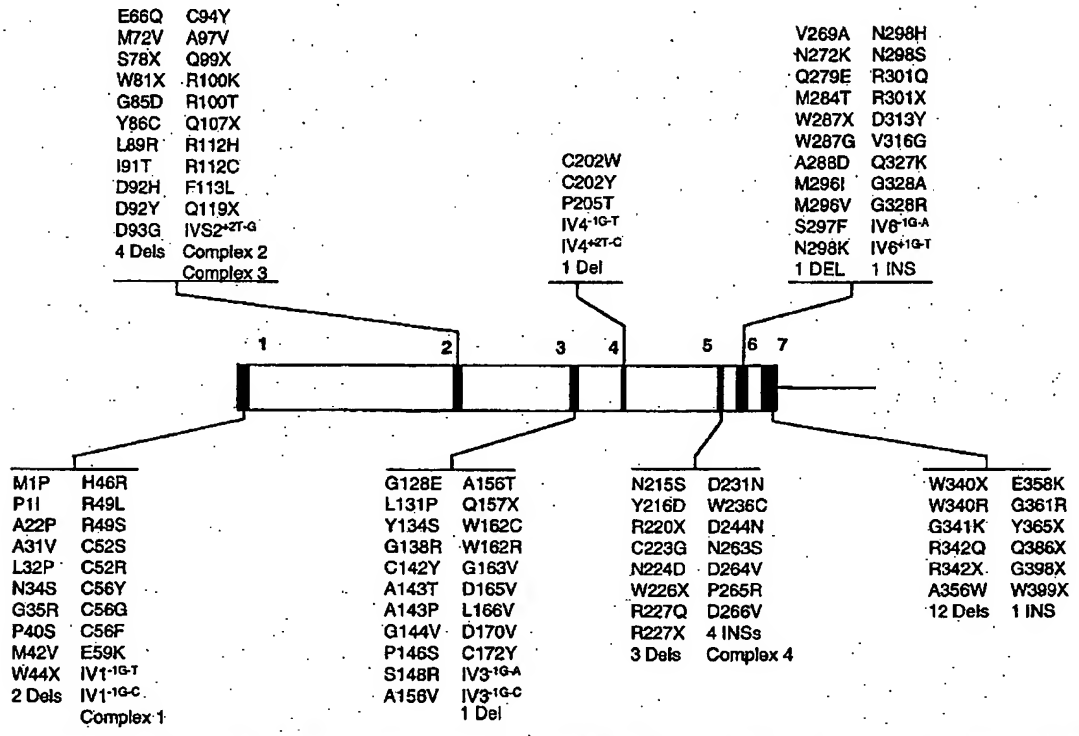

Fig. 150-18 Schematic of the α-galactosidase A gene indicating the relative position of the seven exons and listing the mutations identified in each. Numbers for deletions or insertions refer to nucleotide position in the α-galactosidase A cDNA sequence. Ins = insertion; Δ = deletion; IVS = intervening sequence; see the Human Gene Mutation Database (www.uwcm.ac.uk/uwcm/mg/hgmd0.html) listing for GLA for additional information about the mutations.

α-Galactosidase A Small Insertions and Deletions. In contrast to the rarity of large deletions or duplications, a number of small insertions and deletions have been described in the α-galactosidase A coding sequence in unrelated Fabry patients (see HGMD for list and references). These rearrangements result in either frameshift mutations that lead to premature chain termination or insertion or deletion of one or more amino acids (e.g., 257del18). Of the 30 small insertions and deletions reported to date, 43 percent occurred in exon 7. That a large proportion of the small gene rearrangements occurred in this exon indicates that it is highly susceptible to gene rearrangements and that a mutational "hot spot" may exist in the region of codons 353 to 365.[372] An additional "hot spot" for deletions also was identified in exon 2 from codons 111 to 122.[294]

Complex Mutations. Four complex mutations, which are defined as mutations involving at least two presumably simultaneous mutational events, have been identified in patients with Fabry disease. These occur in exons 1, 2, and 5 of the α-galactosidase A gene.[372,384] In part, these mutations are termed complex because they cannot be readily explained by the usual mutational mechanisms. These mutations are g1312TGCAC → GCTCG in exon 1, g5115GGCAGAGCTCATG → GCAGAGCCA and c356AGCTAGCT → AGCCAACT in exon 2, and c654ATCCGA → AATCGA in exon 5. Complex mutations have been identified in several genes, including the LDL receptor gene,[385] the HPRT gene,[386] and the serum cholinesterase gene.[387] The exon 1 rearrangement resulted from a series of sequence alterations occurring between the tetranucleotide short direct repeat CTGG (normal sequence = CTGG CTGCA CTGG; mutant sequence = CTGG CGCTC GTGG). The α-galactosidase A exon 2 complex rearrangement g5115GGCAGAGCTCATG → GCAGAGCCA involved a series of three small deletions in a 13-bp region (G GCA GAG CTC ATG) from nucleotide 216 to 218. There were a total of four bases deleted and therefore a frameshift was predicted at codon 72.[372] The other exon 2 complex mutation, c356AGCTAGCT → AGCCAACT, resulted from a mutation(s) that altered the underlined two bases in the sequence AGCTAGCT to AGCCAACT. The mutant sequence predicted the in-frame substitution of L120 and A121 to P120 and T121 (L120P and A121T).[384] The complex mutation in exon 5 involved the insertion of an adenosine residue after nucleotide 654 (duplication of the A at position 654) and the deletion of the cytosine at position 656 (ATC CGA to AAT CGA). These base changes predicted an isoleucine to asparagine substitution at position 219 (I219N).[384] Interestingly, the adenosine duplication was preceded by three other adenosine residues (nucleotides 651 to 653). Various mechanisms have been proposed for the generation of these mutations: these include gene conversion between evolutionarily-related sequences[388] or misalignment of "quasi-palindromic" sequences during DNA replication and the subsequent deletion or insertion of bases that might serve to stabilize a hairpin loop.[389] Analysis of the α-galactosidase A sequences in exons 1 and 5 does not support gene conversion as the mutational mechanism for generation of these complex mutations. The existence of several alterations involving multiple insertion and deletion events is unique to the α-galactosidase A gene.

Common Molecular Lesions in Fabry Disease. As noted above, most of the reported mutations have been private (i.e., confined to a single Fabry pedigree). In fact, the discovery that two presumably unrelated families had identical mutations has frequently led to linking of two distant arms of the same pedigree. In contrast, mutations occurring at CpG dinucleotides have been found in unrelated families of different ethnic or geographic backgrounds. Of the 14 CpG dinucleotides in the α-galactosidase A coding sequence, point mutations have been described in eight

(in codons 49, 112, 142/143, 220, 227, 301, 342, and 356); codons 49, 112, 227, 301, and 342 had mutations at both the C and G of their respective CpG dinucleotides. These include R49L, R49S, R112C, R112H, R227Q, R227X, R301Q, R301X, R342Q, and R342X mutations. R227Q and R227X, which occurred at a CpG nucleotide, were the most common mutations causing the classic phenotype. Taken together, they were found in 5 percent (8 of 148) of unrelated Fabry families studied,[294] including families whose mutant alleles could be traced to Danish, English, German, Indian, Irish, Italian, and Polish ancestries. Two families with the R227Q mutation were of German descent, but a common ancestor or demographic region could not be identified. Several apparently unrelated families have had either the R112C or R112H or the R342Q or R342X mutations.[368,369] Therefore, these CpG associated mutations account for the vast majority of recurrent molecular lesions seen in Fabry disease families. The high frequency of mutations at CpG dinucleotides is consistent with their recognition as mutational hot spots due to the deamination of methylcytosine to thymidine.[390] However, N215S, a common mutation among atypical hemizygotes who were asymptomatic or had mild disease manifestations (i.e., cardiac variants), did not occur at a CpG dinucleotide.[119,294,368]

Genotype/Phenotype Correlations

Affected hemizygotes with the classic disease manifestations and no detectable α-galactosidase A activity had a variety of α-galactosidase A lesions, including large and small gene rearrangements, splicing defects, and missense or nonsense mutations. In contrast, all of the asymptomatic or mildly affected atypical hemizygotes (see Table 150-2) had missense mutations that expressed residual α-galactosidase A activity (see "Residual Activity in Atypical Hemizygotes" above). However, efforts to establish genotype/phenotype correlations have been limited, because most Fabry patients had private mutations, and attempts to predict the phenotype require more extensive clinical information from unrelated patients with the same genotype. In addition, attempts to predict the clinical phenotype on the basis of the type or location of a molecular lesion are premature. For example, several atypical mild mutations, N215S, Q279E, M296V, and R301Q, are located in exons 5 and 6. However, other nearby missense mutations, such as S297F, which is adjacent to M296V, result in severe disease. The type of amino acid change (i.e., isofunctional versus altered charge) also fails to predict a classic or mild phenotype. Thus, the clinical severity of private missense mutations detected in Fabry families with few or only young patients is difficult to predict. However, it is anticipated that future crystallographic studies may provide useful structure-function information for genotype/phenotype correlations. Moreover, the influence of modifier genes or other genetic factors on the severity of the disease phenotype may be important since individuals from different pedigrees who share the same mutation and even members of the same family, can have markedly variable phenotypes. In fact, patients from unrelated families with the R112H, R301Q, and G328R mutations have had the classic disease phenotype in one family whereas affected hemizygotes in the other family had mild disease manifestations.[117,126] These occurrences, although rare at present, may become more frequent as more Fabry families are genotyped.

Insights into the structure-function relationships of specific α-galactosidase A amino acids and domains can be gained by noting the position of the point mutations causing classic or variant Fabry disease and their relative conservation in the over 20 homologues encoding α-galactosidase A or the evolutionarily related enzyme α-*N*-acetylgalactosaminidase (α-galactosidase B)[245] currently recorded in the GenBank database. For example, two mutations involving cysteine residues (C94Y and C202Y) were highly conserved in 85 percent and 90 percent of the sequences analyzed, respectively, indicating their probable importance in the formation of intramolecular disulfide bridges in the enzyme polypeptide. In a study of 35 novel α-galactosidase A mutations, several observations were made regarding the location of mutations in conserved regions.[372] In this study, seven missense mutations (Y86C, L89P, I91T, D92Y, C94Y, R100T, and F113L) occurred in exon 2, in a highly conserved region presumed to contain residues in the active site.[365] Six of the seven exon 2 missense mutations were highly conserved in the 20 α-galactosidase A and α-galactosidase B sequences examined (ranging from 80 percent to 95 percent conserved), with the divergence occurring in the bacterial sequences. Moreover, the mutations in two cardiac variants were also located in exon 2 and occurred at moderately to highly conserved residues (I91T, 80 percent; F113L, 85 percent), further demonstrating the functional importance of this region. Other mutations that occurred at highly conserved (> 75 percent) residues were Y134S, S148R, D170V, N263S, and W287C, indicating their importance in the activity and/or stability of the enzyme. Certain mutations occurred in positions that were conserved residues only in mammalian α-galactosidase A sequences (e.g., A31V, H46R, A97V, and L89P). Presumably, substitutions occurring at less essential residues would be neutral or may retain sufficient enzymatic function to be clinically insignificant.

PATHOPHYSIOLOGY

The pattern of glycosphingolipid deposition in Fabry disease, particularly its predilection for vascular endothelial and smooth-muscle cells, is uniquely different from that seen in other glycosphingolipidoses.[391] However, the origin of the accumulated glycosphingolipid substrates has not been fully clarified. A significant contribution comes from endogenous synthesis and subsequent lysosomal accumulation of terminal α-galactosyl containing glycosphingolipids following autophagy of cellular membranous material containing these lipid substrates. Endogenous metabolism is a major source of substrate accumulation in avascular sites such as cornea and in neural cells, which presumably are protected from the increased circulating levels of globotriaosylceramide by the blood-brain barrier. In addition, the turnover of globotriaosylceramide, and particularly its precursor, globotetraosylceramide (globoside), which are present in high concentrations in normal renal tissue, are presumably responsible for the endogenous renal deposition of the Fabry substrates.

The unique cellular and tissue distribution of accumulated globotriaosylceramide, particularly in the vascular endothelium (Fig. 150-19) and smooth muscle, suggests that a significant intracellular contribution may be derived by the endocytosis or diffusion of globotriaosylceramide from the circulation, where the concentration is three- to tenfold higher than in normal individuals. The circulating globotriaosylceramide is primarily transported in the LDL and HDL lipoproteins.[177–179,189,392] In plasma from affected hemizygotes, the accumulated globotriaosylceramide is distributed in the LDL and HDL fractions in proportions similar to those in normal plasma, approximately 60 and 30 percent, respectively. The finding that little, if any, substrate deposition occurs in Fabry hepatocytes (in contrast to the accumulation in Kupffer cells)[141,167,393] supports the contention that globotriaosylceramide synthesized in hepatocytes is associated with the lipoproteins and secreted as a complex.[394] In support of this concept is the fact that patients with hypercholesterolemia have proportional plasma elevations of both LDL and neutral glycosphingolipids, including globotriaosylceramide.[177] The circulating globotriaosylceramide then presumably gains access to vascular endothelial and smooth-muscle cells throughout the body by the high-affinity lipoprotein receptor-mediated uptake pathway.[395–397] Deposits in other tissues may also be derived to a lesser extent from receptor-independent diffusion or by non-absorptive endocytosis of globoside- or globotriaosylceramide-lipoprotein complexes from the plasma. Because lysosomes in all cells are deficient in the α-galactosidase A activity needed to degrade the deposited glycosphingolipids, the glycosphingolipids accumulate within extended multivesicular bodies or, in more